Biochemical Pharmacology, Vol. 27, pp. 943–945. Pergamon Press, 1978. Printed in Great Britain.

EFFECTS OF 2-ARYL-1,3-INDANDIONES ON THE REDUCTION OF 2,6-DICHLOROPHENOL INDOPHENOL BY METHEMOGLOBIN REDUCTASE

J. DE VRIES*, C. N. VERBOOM, L. VAN BREE and W. TH. NAUTA

Department of Medicinal Chemistry, Vrije Universiteit, De Boelelaan 1083, Amsterdam, The Netherlands

(*Received* 22 *August* 1977; *accepted* 19 *October* 1977)

Abstract—Both steric and electronic factors appeared to be essential to the interaction of 2-aryl-1,3-indandiones with methemoglobin reductase. 2-Phenyl-1,3-indandione, 2-(3,5-di-*tert*.butylphenyl)-1,3-indandione and 2-(4-methoxyphenyl)-1,3-indandione were found to be cofactors of the enzyme. By introducing electron-attracting substituents into the phenyl ring strong competitive inhibitors were obtained: 2-(3,5-dichlorophenyl)-1,3-indandione and 2-(3,5-di-trifluoromethylphenyl)-1,3-indandione. Two ortho-substituted derivatives, 2-(2,6-dimethylphenyl)-1,3-indandione and 2-(2,4,6-trimethylphenyl)-1,3-indandione, had no effect at all on the reduction. The anti-inflammatory activities of 2-aryl-1,3-indandiones, as determined in a carrageenan oedema test, showed no relationship to the interaction with the enzyme.

Non-steroidal anti-inflammatory drugs have been reported to affect cellular oxidation–reduction reactions [1]. The influence on two oxidation processes, uncoupling of oxidative phosphorylation and inhibition of prostaglandin biosynthesis, has frequently been used to distinguish between anti-inflammatory and non-anti-inflammatory compounds. However, anti-inflammatory activity does not always correlate with the *in vitro* activities. This has also been described for a series of 2-aryl-1,3-indandiones by Van den Berg *et al.* [2, 3].

In a previous study on a reductive enzyme system we have met the same problem [4]. In the reduction of 2,6-dichlorophenol indophenol (DCIP) by methemoglobin reductase 2-phenyl-1,3-indandione (PID) and phenylbutazone have been found to act as cofactors, and sodium salicylate and indometacin as inhibitors. Methemoglobin reductase (a diaphorase) is located in the erythrocyte and regulates the correct hemoglobin–methemoglobin ratio [5].

In an effort to clarify the above problem we have studied the influence on methemoglobin reductase by seven selected 2-aryl-1,3-indandiones—PID, 2-(2,6-dimethylphenyl)-1,3-indandione, 2-(2,4,6-trimethylphenyl)-1,3-indandione, 2-(3,5-di-*tert*.butylphenyl)-1,3-indandione, 2-(4-methoxyphenyl)-1,3-indandione, and 2-(3,5-dichlorophenyl)-1,3-indandione and 2-(3,5-di-trifluoromethylphenyl)-1,3-indandione and three well-known anti-inflammatory compounds, sodium salicylate, phenylbutazone and indometacin.

MATERIALS AND METHODS

Chemicals. The 2-aryl-1,3-indandiones came from the laboratory stock. All other chemicals were of analytical grade and were purchased from J. T. Baker Chemicals B.V., Deventer, Netherlands.

* To whom correspondence should be addressed.

Preparation of the enzyme. The enzyme was isolated from bovine erythrocytes according to Splittgerber *et al.* [6]. Protein was determined by the method of Lowry *et al.* [7].

Enzyme assay. The methemoglobin reductase activity was measured according to the method of Scott and McGraw [8], using DCIP as the electron acceptor. The assay system consisted of 45 mM Tris–HCl buffer (pH = 7.5), 1 mM EDTA and 9–59 μM DCIP (determined in the cuvette by using a millimolar extinction coefficient of 20.1); the protein concentration was 125 μg/ml. When NADH was used, its concentration was 133 μM. The drugs were added in 0.05 ml of DMSO. The reactions were started by the addition of the cofactor. In the inhibition studies the inhibitor was added just before the reaction was started. The initial reduction velocities were measured at 20° with a Beckman 24 recording spectrophotometer at 600 nm. The non-enzymatic reduction of DCIP by NADH, the 2-aryl-1,3-indandiones, sodium salicylate, phenylbutazone and indometacin was corrected for, if it was less than 5 per cent of the final rate.

RESULTS AND DISCUSSION

The 2-aryl-1,3-indandiones have mainly been selected, starting from the way in which their anti-inflammatory activities correlate with their effects on uncoupling of oxidative phosphorylation and prostaglandin biosynthesis, as observed by Van den Berg *et al.* [2, 3] (Table 1). For the influence on both biochemical processes marked differences were found between 2-aryl-1,3-indandiones with and without ortho substituents. In the case of uncoupling of oxidative phosphorylation 3,5-di-*tert*.butylphenylindandione appeared to be one of the most active agents. PID was a weak inhibitor of prostaglandin biosynthesis but by introducing electron-

Table 1. Inhibition of the development of carrageenan oedema in the hind paw of the rat (ED_{50}, mg/kg)* by a number of 2-(R-phenyl)-1,3-indandiones and three non-steroidal anti-inflammatory compounds and their effects on oxidative phosphorylation (C_{50}, M), prostaglandin biosynthesis (ID_{50}, M) and reduction of DCIP by methemoglobin reductase

R. or compound	ED_{50}	$-\log C_{50}$	$-\log ID_{50}$	Effect on meth. red.
H	37	3.74	3.22	cofactor/non-enz.red. (< 5%)
2,6-dimethyl	—†	3.45	< 3.20	—
2,4,6-trimethyl	—	3.60	< 3.20	—
3,5-di-*tert*.butyl	5.4	5.61	4.95	cofactor/non-enz.red.
4-methoxy	77	3.75	3.37	cofactor/non-enz.red.
3,5-dichloro	—	4.31	5.19	comp. inhibition
3,5-di-trifluoromethyl	36.6‡	N.D.§	N.D.	comp. inhibition
sodium salicylate	310	2.62	< 3.00	comp. inhibition
phenylbutazone	46	3.15	3.40	cofactor
indometacin	3.6	3.54	5.00	comp. inhibition

* The compounds were given orally.
† No activity observed.
‡ See [9].
§ Not determined.

attracting substituents very active compounds were obtained as 3,5-dichlorophenylindandione.

4-Methoxyphenylindandione, another derivative with an electron-repelling group, and 3,5-di-trifluoromethylphenylindandione, another derivative with an electron-attracting group, have been added to the series in view of the fact that PID can act as an electron donor in the reduction of DCIP by methemoglobin reductase [4].

The 3,5-di-*tert*.butyl- and 4-methoxyphenyl-indandiones can also act as cofactors in the enzymatic reduction of DCIP. In these cases it was not possible to determine the initial reduction velocities, because the non-enzymatic reduction of DCIP by the indandiones was far more than 5 per cent of the final rate.

However, replacement of NADH as a cofactor of methemoglobin reductase in the reduction of DCIP is not a general property of 2-aryl-1,3-indandiones. The 3,5-dichloro- and 3,5-di-trifluoromethyl derivatives were not able to replace NADH as a cofactor and did not show non-enzymatic reduction of DCIP.

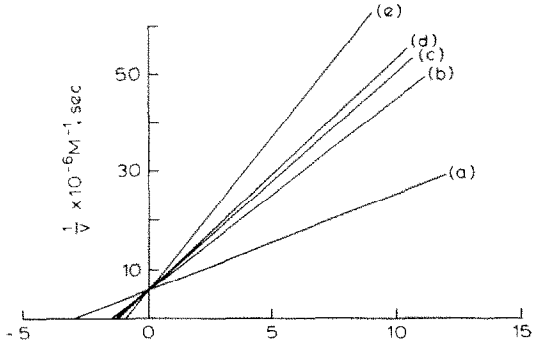


Fig. 1. Lineweaver–Burk plots of the reduction of DCIP by methemoglobin reductase in the presence of 133 μM NADH (a), of 133 μM NADH+0.05 mM 3,5-dichlorophenylindandione (b), of 133 μM NADH+0.05 mM 3,5-di-trifluoromethylphenylindandione (c), of 133 μM NADH+20 mM sodium salicylate (d) and of 133 μM NADH+0.5 mM indometacin (e). Each point represents the mean value of at least three experiments.

On the contrary, both indandiones were competitive inhibitors of the enzyme (Fig. 1).

The kinetic expression for this type of inhibition is

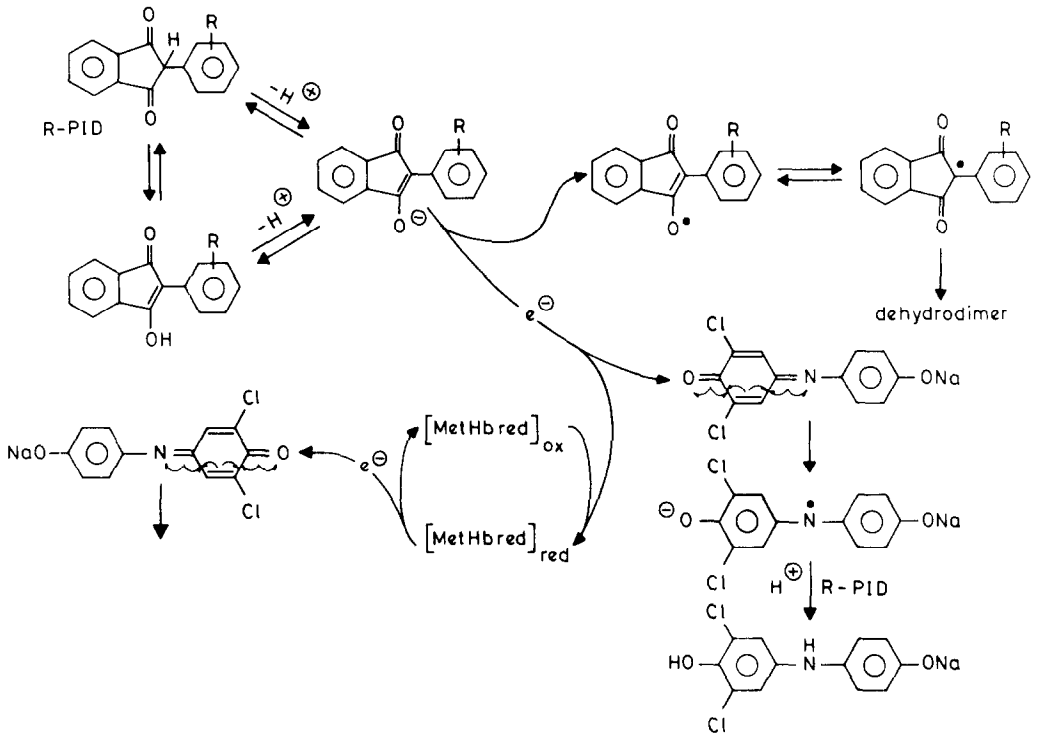
$$\frac{V_{max}}{v} = \frac{K_m}{[S]} + \frac{K_m \cdot [I]}{[S] \cdot K_i} + 1$$

The following inhibitory constants were calculated: 52 and 49 μM, respectively. The 2,6-dimethyl and 2,4,6-trimethyl derivatives had no effect at all on the reduction neither as electron donors nor as inhibitors. The electron-donating ability of 2-aryl-1,3-indandiones might be explained as follows. Considering the charge distribution over the five-membered ring of the indane skeleton, abstraction of a hydride ion like in the case of NADH is not likely. Zalukaev *et al.* [10] reported the reduction of quinones by 2-aryl-1,3-indandiones. They described that cyclic β-diketones react in the anionic form with the quinone by a mechanism of one-electron transfer. We postulate that also in the reduction of DCIP by methemoglobin reductase using a 2-aryl-1,3-indandione as a cofactor and in the non-enzymatic reduction of DCIP by the same arylindandione the first step is an one-electron transfer (Scheme 1). The anion radical can be stabilised by delocalisation of the unshared electron over the phenyl group. Such a stabilisation can not occur in the 2,6-dimethyl- and 2,4,6-trimethylphenylindandiones, because the five-membered ring of the indane group and the phenyl group are not coplanar, as shown by Bruynes [11] in a nuclear magnetic resonance study.

The absence or presence of the electron-donating ability in the case of the other 2-aryl-1,3-indandiones becomes clear by using the Hammett σ constant of the relevant substituent as a parameter for the π-electron availability of carbon atom 1 of the phenyl group.

In support of the above postulate are the results of an electron spin resonance study on 2-aryl-1,3-indandione radicals by Poluktov *et al.* [12]. They observed that 2-(4-dimethylaminophenyl)-1,3-indandione formed a stable radical. The σ_{para} of a dimethylamino group is -0.600 [13].

In accordance with the negative σ values of para



Scheme 1. Reduction of DCIP by methemoglobin reductase using a 2-(R-phenyl)-1,3-indandione as a cofactor and non-enzymatic reduction of DCIP by the same indandione, postulated mechanisms.

methoxy (−0.268 [13]) and meta *tert.*-butyl (−0.240 [13]) groups, the 4-methoxy and 3,5-di-*tert.*butyl derivatives were found to act as electron donors in the enzymatic and non-enzymatic reduction of DCIP. As might be expected on the ground of the fact that the σ value of a hydrogen atom is zero, the non-enzymatic reduction of DCIP by the unsubstituted phenylindandione was less extensive [4]. The σ_{meta} values of chloro (0.746 [13]) and trifluoromethyl (0.860 [13]) substituents are positive and the corresponding phenylindandiones were no electron donors. It is evident from the results reported above that the interaction with methemoglobin reductase or the ability to form radicals is not a right tool to distinguish between anti-inflammatory and non-anti-inflammatory 2-aryl-1,3-indandiones (Table 1). The same applies to the reference compounds sodium salicylate, phenylbutazone and indometacin. Sodium salicylate and indometacin were competitive inhibitors of methemoglobin reductase (see Fig. 1. K_i = 15 mM and 230 μM respectively) while Verboom *et al.* [4] demonstrated that phenylbutazone acted as a cofactor of the enzyme.

Finally, however, it should be pointed out that representatives of two well-known classes of drugs, the anticoagulants and the anti-inflammatories, may affect the hemoglobin–methemoglobin ratio, in other words the oxygen transport.

REFERENCES

1. M. W. Whitehouse, *Prog. Drug Res.* **8**, 321 (1965).
2. G. van den Berg and W. Th. Nauta, *Biochem. Pharmac.* **24**, 815 (1975).
3. G. van den Berg, T. Bultsma and W. Th. Nauta, *Biochem. Pharmac.* **24**, 1115 (1975).
4. C. N. Verboom, J. de Vries, L. van Bree and W. Th. Nauta, *Biochem. Pharmac.* **26**, 685 (1977).
5. E. R. Jaffé and G. Neumann, in *Proceedings of the First International Symposium on Metabolism and Membrane Permeability of Erythrocytes and Thrombocytes* p. 83. Verlag Stuttgart (1968).
6. A. G. Splittgerber, K. Mitchell, G. Dahle, M. Puffer and K. Blomquist, *J. Chem. Educ.* **52**, 680 (1975).
7. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
8. E. M. Scott and J. C. McGraw, *J. biol. Chem.* **237**, 249 (1962).
9. J. Ch. Eriks, H. van der Goot, O. P. Zuiderveld and W. Th. Nauta, (to be published).
10. L. P. Zalukaev, V. V. Krivosheni and G. A. Kharina, *Zhur. Obshsch. Kim.* **34**, 2478 (1964).
11. C. A. Bruynes, *Ph.D. Thesis*, Vrije Universiteit, Amsterdam (1968).
12. I. T. Poluktov, V. V. Moiseev, I. F. Gainulin and A. I. Yasmenko, *Zhur. Org. Kim.* **11**, 1300 (1975).
13. H. Jaffé, *Chem. Rev.* **53**, 191 (1953).